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1. Kliewer et al. (1998) Cell 92: 73-82.
2. Zhang et al. (1999) Arch. Biochem. Biophys. 368: 14-22.
3. Li et al. (1997) Endocrinology 138: 2347-2353.

An Orphan Nuclear Receptor Activated by Pregnanes Delineates a Novel Steroid Signaling Pathway

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Summary

Steroid hormones exert profound effects on differentiation, development, and homeostasis in higher eukaryotes through interactions with nuclear receptors. We describe a novel orphan nuclear receptor, termed the pregnane X receptor (PXR), that is activated by naturally occurring steroids such as pregnenolone and progesterone, and synthetic glucocorticoids and anti-glucocorticoids. PXR exists as two isoforms, PXR.1 and PXR.2, that are differentially activated by steroids. Notably, PXR.1 is efficaciously activated by pregnenolone 16α-carbonitrile, a glucocorticoid receptor antagonist that induces the expression of the CYP3A family of steroid hydroxylases and modulates sterol and bile acid biosynthesis in vivo. Our results provide evidence for the existence of a novel steroid hormone signaling pathway with potential implications in the regulation of steroid hormone and sterol homeostasis.

Introduction

Steroid hormones are cholesterol derivatives that serve as signaling molecules to coordinate the expression of complex gene programs in higher eukaryotes. These molecules exert their effects by diffusing into cells and interacting with specific intracellular receptors. Receptors for each of the major classes of sex and adrenal steroids have been characterized (Evans, 1988; Beato et al., 1995; Mangelsdorf et al., 1995). In the absence of their cognate ligands, the steroid hormone receptors remain sequestered in the cytoplasm through interactions with large multiprotein complexes containing heat shock proteins. However, the binding of ligand causes the steroid hormone receptors to be released from these complexes and translocated into the nucleus (Pratt,

1993). Once inside the nucleus, the activated receptors regulate the expression of target genes by binding as homodimers to short DNA sequence motifs, termed hormone response elements (HREs) (Glass, 1994). In this manner, the steroid hormone receptors function as ligand-activated transcription factors.

The molecular cloning of steroid hormone receptors revealed that they comprise a subfamily within a larger superfamily of structurally related proteins (Evans, 1988; Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995). This superfamily also includes receptors for nonsteroidal, lipophilic molecules such as thyroid hormone, retinoids, fatty acids, and eicosanoids. The nonsteroid receptors differ from their steroid hormone receptor counterparts in several respects (Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995). First, the nonsteroid hormone receptors are not sequestered in the cytoplasm in the absence of their cognate ligands but instead reside within the nucleus. Second, whereas steroid hormone receptors generally bind to their HREs as homodimers, most of the nonsteroid hormone receptors identified to date bind to DNA as heterodimers with the 9-cis retinoic acid receptors (RXRs) (Glass, 1994; Mangelsdorf and Evans, 1995). Finally, the two classes of receptors recognize different types of HREs: steroid hormone receptors generally bind to HREs composed of two half-sites organized as a palindrome with a three-nucleotide spacer, while nonsteroid hormone receptors preferentially bind to HREs composed of two half-sites organized as a direct repeat (DR), with the number and composition of the nucleotides separating the half-sites serving as important determinants of receptor selectivity (Umesono et al., 1991; Glass, 1994; Mangelsdorf and Evans, 1995).

In addition to receptors with established ligands, approximately 30 other members of the nuclear hormone receptor family have been isolated from vertebrates. These related proteins lack identified ligands and, as a consequence, have been termed orphan nuclear receptors (Evans, 1988; Mangelsdorf and Evans, 1995; Enmark and Gustafsson, 1996). The search for hormonal activators of the orphan receptors has created exciting opportunities to discover novel endocrine signaling pathways with implications in both normal physiology and disease. Work with orphan nuclear receptors has led to the identification of fatty acids and eicosanoids as ligands for the peroxisome proliferator-activated receptors (Forman et al., 1997; Kliewer et al., 1997; Krey et al., 1997), retinoids and farnesoids as activators of RXR1/FXR (Forman et al., 1995; Zavacki et al., 1997), and various oxysterols as activators of the LXR and SF-1 orphan nuclear receptors (Janowski et al., 1996; Lala et al., 1997; Lehmann et al., 1997).

In this report, we describe a novel orphan nuclear receptor, which we have designated PXR for pregnane X receptor. Like nonsteroid hormone receptors, PXR binds as a heterodimer with RXR to an HRE composed of two half-sites organized as a DR. Surprisingly, however,

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PXR is efficaciously activated by several steroids, including naturally occurring pregnanes and synthetic glucocorticoids and antiglucocorticoids. Thus, PXR combines features of both the steroid and nonsteroid nuclear receptors. We suggest that PXR defines a novel steroid hormone signalling pathway that may account for at least some of the effects of synthetic glucocorticoids and antiglucocorticoids that do not appear to be mediated through the classical glucocorticoid receptor (GR) signalling pathway.

Results

Cloning of PXR.1 and PXR.2

In an effort to identify new members of the nuclear receptor family, we performed a series of motif searches of public EST databases. These searches revealed a clone from a mouse liver library in the Washington University/HMMI EST database that had homology to the ligand-binding domains (LBDs) of a number of nuclear receptors. We used this partial sequence information to isolate larger clones from a mouse liver cDNA library. The nucleotide sequence of the longest cDNA clone encodes a novel orphan nuclear receptor of 431 amino acids that we have designated PXR.1 (Figure 1A). We also isolated a second cDNA clone, termed PXR.2, that was identical to PXR.1 except for the deletion of a stretch of 123 nucleotides extending from base pairs 661 to 783 (Figure 1A). Examination of the PXR genomic structure revealed that PXR.2 represents a splice variant of PXR.1 lacking a single exon (data not shown). The PXR.2 cDNA encodes a 390 amino acid protein that lacks a 41 amino acid region in the putative LBD of PXR.1. Sequence alignment with nuclear receptors for which the crystal structures have been solved indicates that the 41 amino acids that distinguish the PXR isoforms lie between helices two and three of the canonical LBD structure (Wurtz et al., 1996).

Sequence comparison with other members of the nuclear receptor family showed that the PXR isoforms are most closely related to the *Xenopus laevis* orphan nuclear receptor 1 (ONR1) (Smith et al., 1994), with PXR.2 and ONR1 sharing 70% and 46% amino acid identity in their DNA-binding domains (DBDs) and LBDs, respectively (Figure 1B). Based upon this degree of homology, it is unclear at present whether PXR represents the mammalian homolog of ONR1. With regard to mammalian receptors, PXR is most closely related to the vitamin D receptor (VDR) (Baker et al., 1988), sharing 64% and 39% identity in the DBD and LBD, respectively.

PXR Expression Pattern in the Embryo and the Adult

The expression pattern of PXR was examined in both adult and embryonic tissues. Northern blot analysis was performed under high-stringency conditions with blots that included poly(A)⁺ RNA prepared from multiple adult mouse tissues and a probe that recognized both PXR isoforms. Abundant expression of PXR mRNA was observed only in the liver and intestine, where three distinct messages were detected including a highly expressed mRNA species of 2.6 kb and two transcripts of 1.9 kb

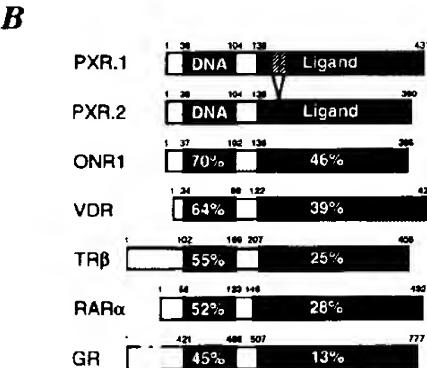
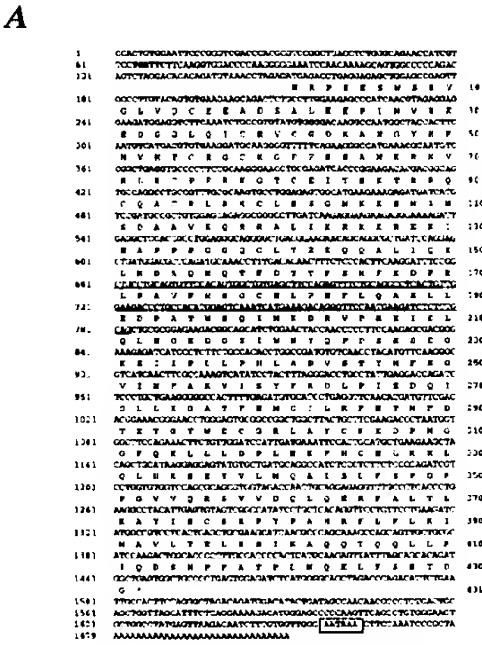


Figure 1. PXR is a Member of the Nuclear Receptor Superfamily
(A) Nucleotide and predicted amino acid sequence of mouse PXR. The upstream in-frame stop codon is in bold. The predicted start initiation codon is at nucleotide 151. The 123 nucleotide region extending from base pairs 661 to 783 that is present in PXR.1 and absent in PXR.2 is underlined. A putative polyadenylation signal in the 3' untranslated region is boxed.
(B) Amino acid sequence comparison between murine PXR and other members of the nuclear hormone receptor family. Similarity between PXR and other nuclear hormone receptor family members in the DNA- and ligand-binding domains are indicated as percent amino acid identity. The 41 amino acid region that distinguishes PXR.1 from PXR.2 is indicated by the cross-hatched area. All comparisons were made with the PXR.2 isoform. ONR1, *Xenopus* orphan nuclear receptor 1; VDR, human vitamin D receptor; TR β , human thyroid hormone receptor β ; RAR α , human retinoic acid receptor α ; GR, human glucocorticoid receptor.

and 4.4 kb that were expressed at lower levels (Figure 2A). Weaker expression of the *PXR* mRNA was also detected in kidney and stomach (Figure 2A). No *PXR* mRNA was detected in the other tissues examined.

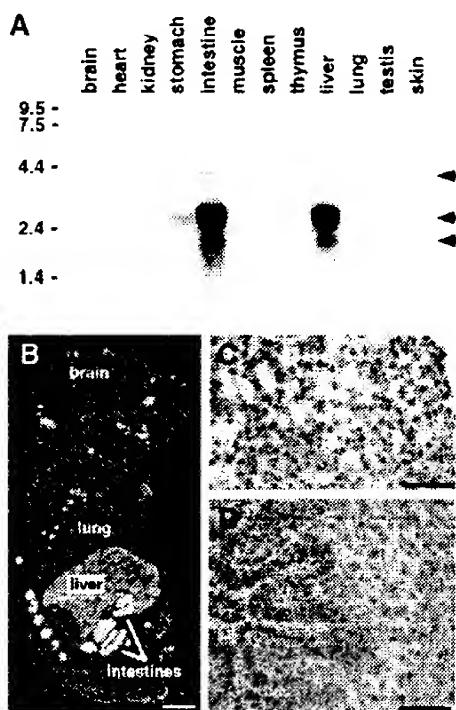


Figure 2. Expression Pattern of PXR in Adult and Embryonic Tissues
(A) Northern blot analysis. RNA size markers (in kb) are indicated at left. Arrows at right indicate the three species of PXR transcripts that were detected. (B-D) In situ hybridization analysis of PXR mRNA expression in E18 mouse embryo sections with a probe that recognized both PXR isoforms (see probe 1 in Experimental Procedures). (B) Phosphoimage of an entire sagittal section showing specific labeling in intestine and liver. Specificity was ascertained by competition with a 100-fold excess of unlabeled, specific oligonucleotide. This resulted in complete inhibition of labeling in liver and intestine while nonspecific signals remained in developing bone. (C and D) High power bright-field microscopy showing the abundance of silver grains in the liver (C) and in the intestine (D) where mRNA labeling was confined to the epithelium. The bar in (B) corresponds to 2 mM, and in (C) and (D) to 50 μ M.

The embryonic expression pattern of PXR was examined via in situ hybridization analysis using sections prepared from day 18 (E18) mouse embryos. Three oligonucleotides were designed that recognized PXR,

start with both PXR isoform only to PXR.1 mRNA. Liver and intestine (Figure 2) expression was confined to PXR expression was detected in brain, lung, thymus, heart, and cord (data not shown). Competition with all three PXR isoforms was performed with the PXR isoforms (data not shown). PXR.2 is expressed in tissues conclude from the Northern analyses that PXR is abundantly expressed in a few tissues, including the mouse embryo and adult.

PXR Is Activated by Synthetic Pregnanes and Glucocorticoids

We next sought to determine whether PXR, like other members of the nuclear receptor family, possesses transcriptional activity that can be regulated in a hormone-dependent manner. As we lacked knowledge of a cognate HRE for PXR, we initially performed searches for activators using an established chimera system in which the LBDs of the two PXR isoforms were fused to the DBD of the yeast transcription factor GAL4 (Lehmann et al., 1995). Expression vectors for the GAL4-PXR chimeras were transiently transfected into CV-1 cells together with a reporter plasmid containing five copies of a GAL4 DNA binding site upstream of the chloramphenicol acetyltransferase (CAT) reporter. Transfected CV-1 cells were systematically treated with a series of natural and synthetic compounds that included steroids, vitamin D analogs, thyroid hormone analogs, retinoids, fatty acids, and other small, lipophilic molecules, and reporter levels measured.

Interestingly, we found that the activity of the GAL4-PXR.1 chimera was markedly induced by 10 μ M concentrations of a variety of synthetic steroids including the glucocorticoids dexamethasone, dexamethasone-t-butyl-acetate, and dexamethasone-21-acetate, and the pregnenolone derivative 6,16 α -dimethyl pregnenolone (Figure 3B). Dexamethasone-t-butylacetate and 6,16 α -dimethyl pregnenolone were the most efficacious of these compounds (Figure 3B). Remarkably, we found that PXR.1 was not only activated by GR agonists, but also by the GR antagonists RU486 and pregnenolone 16 α -carbonitrile (PCN) (Figure 3B). RU486 is a potent antiprogestin that binds to the GR at nanomolar concentrations (Cadepond et al., 1997). PCN is a weaker antagonist that has been shown to interfere with GR-mediated activation at micromolar concentrations (Schuetz and Guzelian, 1984; Schuetz et al., 1984). Thus, the LBD of PXR.1 was efficaciously activated by both agonists and antagonists of the GR.

Notably, the GAL4-PXR.2 chimera displayed a much more restricted activation profile. Of the steroids that activated GAL4-PXR.1, only dexamethasone-t-butyl-acetate activated GAL4-PXR.2 efficiently (Figure 3B). We conclude that the 41 amino acid deletion that distinguishes PXR.2 from PXR.1 has a marked effect on the responsiveness of the orphan receptor to synthetic steroids.

PXR Functions through a Response Element Conserved in the CYP3A Gene Promoters

PCN and dexamethasone treatment have previously been shown to induce the expression of the CYP3A family of genes in rodent liver, intestine, and kidney as well as in primary cultures of rodent hepatocytes (Elshourbagy and Guzelian, 1980; Heuman et al., 1982; Hardwick et al., 1983; Schuetz and Guzelian, 1984; Schuetz et al., 1984; Gonzalez et al., 1985; Debri et al., 1995). The CYP3A genes encode cytochrome P450 hemoproteins involved in the hydroxylation of steroid hormones, including corticosteroids, progestins, androgens, and DHEA-sulfate, as well as a variety of xenobiotics (Nebert and Gonzalez, 1987; Juchau, 1990). The response to PCN and dexamethasone occurs at the level

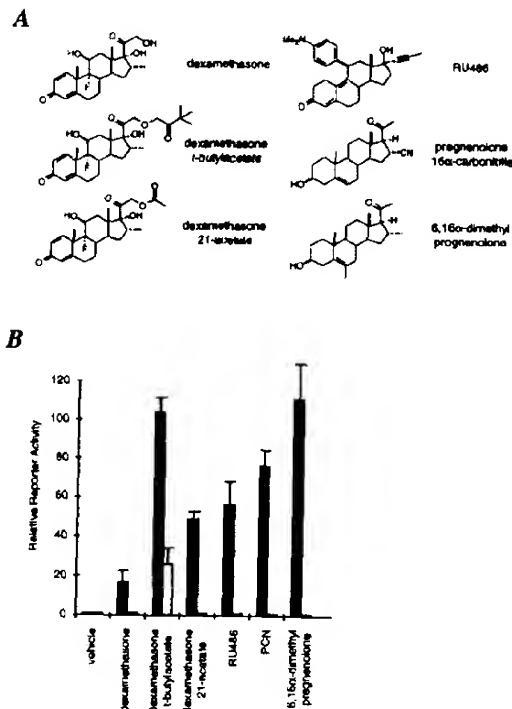


Figure 3. Synthetic Glucocorticoids and Pregnenolone Derivatives Activate PXR

(A) Structures of the steroids that activate the GAL4-PXR chimeric proteins.
(B) CV-1 cells were cotransfected with expression plasmids encoding either GAL4-PXR.1 (filled bars) or GAL4-PXR.2 (open bars) and the reporter plasmid (*luc*-tk-CAT). Cells were treated with vehicle alone (0.1% DMSO) or 10 μ M of the indicated steroids. Cell extracts were subsequently assayed for CAT activity. Data represent the mean of five data points from two different experiments \pm SD.

of transcription and has been mapped to a conserved region within the *CYP3A1* and *CYP3A2* gene promoters that does not contain a typical glucocorticoid response element, but instead contains a DR of the nonsteroid nuclear receptor half-site sequence AGTTCA separated by a three-nucleotide spacer, a so-called DR-3 motif (Figure 4A) (Umesono et al., 1991; Miyata et al., 1995; Quattrochi et al., 1995; Huss et al., 1996). These data have led to speculation that a nonsteroid nuclear hormone receptor might be involved in mediating the effects of PCN and dexamethasone.

Given that PXR shares a high degree of homology with ONR1 and VDR in the DBD (Figure 1B) and that both ONR1 and VDR preferentially bind to DR-3 HREs as heterodimers with RXR (Umesono et al., 1991; Smith et al., 1994; Mangelsdorf and Evans, 1995), we postulated that PXR might bind to the *CYP3A1* and *CYP3A2* DR-3 motifs as a heterodimer with RXR. In order to test this idea, gel mobility shift assays were performed using a radiolabeled oligonucleotide containing the *CYP3A1* DR-3 motif and in vitro synthesized PXR.1, PXR.2, and RXR α . Neither PXR nor RXR α bound to the *CYP3A1* DR-3 alone. However, both PXR.1 and PXR.2 bound

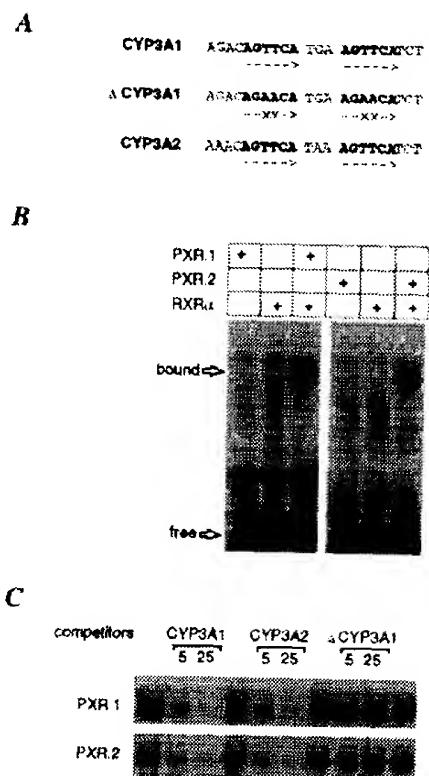


Figure 4. PXR Binds as a Heterodimer with RXR α to DR-3 Response Elements Present in the *CYP3A1* and *CYP3A2* Gene Promoters

(A) Alignment of DR-3 motifs present in the promoter regions of the *CYP3A1* and *CYP3A2* genes. A mutated derivative of the *CYP3A1* DR-3 motif (Δ CYP3A1) used in the gel mobility shift assays and the position of the mutations is also shown.
(B) Gel mobility shift assays were performed with a radiolabeled oligonucleotide containing the *CYP3A1* DR-3 and in vitro synthesized PXR.1, PXR.2, and RXR α as indicated.
(C) Gel mobility shift assays were performed with PXR.1 and RXR α (top panel) or PXR.2 and RXR α (bottom panel) and radiolabeled oligonucleotide containing the *CYP3A1* DR-3 in the presence of either a 5-fold or 25-fold excess of unlabeled oligonucleotides containing the *CYP3A1* DR-3, a mutated *CYP3A1* DR-3 (Δ CYP3A1), or the *CYP3A2* DR-3 as indicated.

efficiently to the *CYP3A1* DR-3 as heterodimers with RXR α (Figure 4B). The PXR-RXR α complex with DNA was competed efficiently by an excess of unlabeled *CYP3A1* DR-3 oligonucleotide or an oligonucleotide containing a closely related DR-3 motif from the *CYP3A2* gene promoter (Figures 4A and 4C). No competition was seen with an oligonucleotide containing a mutated *CYP3A1* DR-3 motif (Figures 4A and 4C). Thus, both PXR isoforms can bind specifically as heterodimers with RXR α to DR-3 motifs found in the promoter regions of *CYP3A* genes.

We next asked whether the PXR isoforms could induce gene expression through the *CYP3A1* DR-3 element in response to steroids. Transient transfection assays were performed with a reporter plasmid containing two copies of the *CYP3A1* DR-3 motif inserted

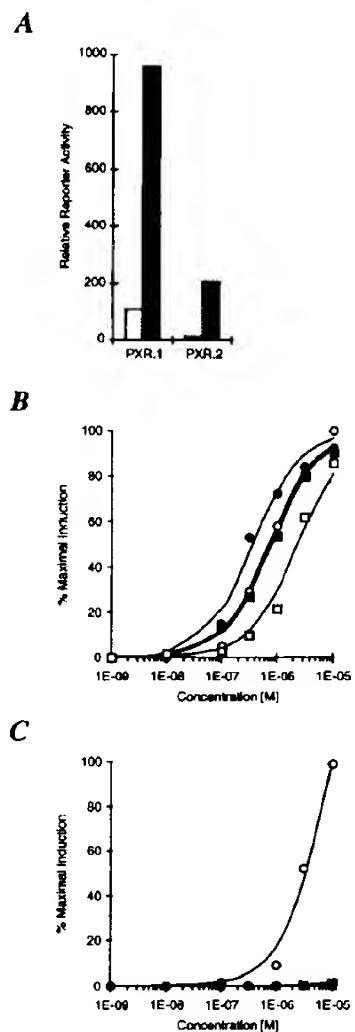


Figure 5. Synthetic Glucocorticoids and Pregnenolone Derivatives Activate PXR through the CYP3A1 DR-3

(A) CV-1 cells were cotransfected with expression plasmids for full-length PXR.1 or PXR.2 and the (CYP3A1)₂-tk-CAT reporter plasmid. Cells were treated with either vehicle (0.1% DMSO) alone (open bars) or 10 μ M dexamethasone-t-butylacetate (filled bars). Cell extracts were subsequently assayed for CAT activity.

(B and C) CV-1 cells were cotransfected with expression plasmids for full-length PXR.1 (B) or PXR.2 (C) and the (CYP3A1)₂-tk-CAT reporter plasmid. Transfected cells were treated with the indicated concentrations of 6,16 α -dimethyl pregnenolone (closed circles), dexamethasone-t-butylacetate (open circles), PCN (closed squares), or RU486 (open squares). Cell extracts were subsequently assayed for CAT activity and data plotted as the percentage of the maximal response obtained. Data points represent the mean of assays performed in triplicate. Similar results were obtained in two independent experiments.

upstream of the minimal thymidine kinase promoter and the CAT gene [(CYP3A1)₂-tk-CAT]. Interestingly, in the absence of steroids, PXR.1 was found to have a roughly 20-fold higher basal level of activity than PXR.2 (Figure 5A). Nevertheless, both PXR isoforms were efficiently

activated by dexamethasone-t-butylacetate (Figure 5A). Dose-response analysis revealed dexamethasone-t-butylacetate to be a significantly more potent activator of PXR.1 than PXR.2, with EC₅₀ values of 0.8 and 5 μ M for PXR.1 and PXR.2, respectively (Figures 5B and 5C). Consistent with the results obtained using the GAL4-PXR chimeras, 6,16 α -dimethyl pregnenolone, PCN, and RU486 activated full-length PXR.1 on the CYP3A1 response element but failed to activate full-length PXR.2 (Figures 5B and 5C). 6,16 α -dimethyl pregnenolone was the most potent of these compounds, activating PXR.1 with an EC₅₀ value of 300 nM (Figure 5B). Based upon these data, we conclude that the full-length PXR isoforms can activate gene expression through the CYP3A1 DR-3 motif in response to synthetic steroids.

PXRs Are Activated by Naturally Occurring Steroids

As PXR was activated by synthetic steroids, we examined whether a naturally occurring steroid might serve as the endogenous hormone for PXR. Accordingly, CV-1 cells were transfected with expression plasmids for either of the full-length PXR isoforms and the (CYP3A1)₂-tk-CAT reporter and treated with a variety of natural steroids, including progestins, glucocorticoids, mineralocorticoids, androgens, estrogens, bile acids, and oxysterols. Both PXR.1 and PXR.2 were activated by micromolar concentrations of certain of these steroids. Consistent with our findings that the pregnenolone derivatives 6,16 α -dimethyl pregnenolone and PCN activate PXR.1, pregnenolone and its metabolites 17 α -hydroxypregnenolone, progesterone, 17 α -hydroxyprogesterone, and 5 β -pregnane-3,20-dione activated PXR.1 (Figure 6B). Dose-response analysis revealed that all five of these pregnanes activated PXR.1 with EC₅₀ values in the 5–20 μ M range (Figure 6C). Since PXR.1 is activated by synthetic glucocorticoids, we were surprised to find that naturally occurring glucocorticoids, including cortisol and corticosterone, had virtually no effect on PXR.1 activity (data not shown).

In analogous cotransfection experiments performed with PXR.2, only 5 β -pregnane-3,20-dione was found to induce reporter activity >5-fold (Figure 6B). Notably, pregnenolone, progesterone, and their 17 α -hydroxylated derivatives, which were efficacious activators of PXR.1, had little or no activity on PXR.2 (Figure 6B). Thus, while both PXR isoforms are activated by naturally occurring pregnanes, PXR.1 is activated by a broader range of these steroids than PXR.2. The generation of LBD isoforms with distinct activation profiles provides a novel mechanism for increasing the regulatory diversity in the PXR signaling pathway.

Steroids Promote the Interaction of PXR with a Coactivator Protein

We next sought to address whether the steroids that activated PXR did so through direct interactions with the LBD. Due to the lack of radiolabeled derivatives of the more potent PXR activators, we were unable to perform standard binding analyses. However, a number of laboratories have recently demonstrated that ligands

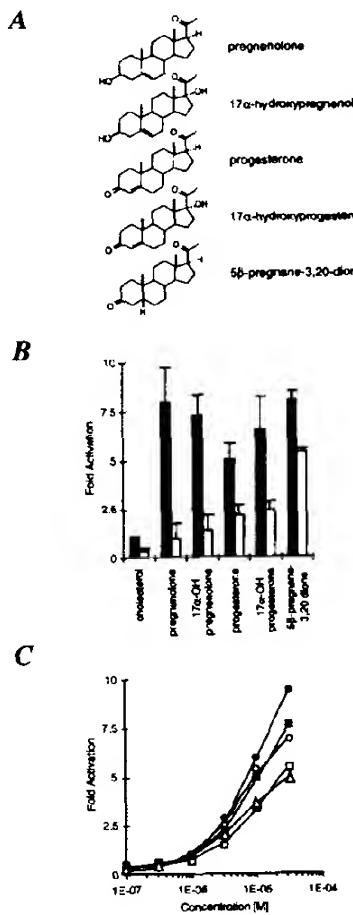


Figure 6. Naturally Occurring Steroids Activate PXR

(A) Structures of naturally occurring steroids that activate PXR.

(B) CV-1 cells were cotransfected with expression plasmids for full-length PXR.1 (filled bars) or PXR.2 (open bars) and the (*CYP3A7*)-*tk-CAT* reporter plasmid. Transfected cells were treated with 10 μ M of the indicated steroids. Cell extracts were subsequently assayed for CAT activity and data plotted as fold-activation relative to cells treated with vehicle (0.1% DMSO) alone. Data points represent the mean of assays performed in triplicate \pm SD.

(C) Transfected cells were treated with the indicated concentrations of pregnenolone (closed circles), 17 α -hydroxypregnенolone (open circles), progesterone (triangles), 17 α -hydroxyprogesterone (open squares), or 5 β -pregnane-3,20-dione (closed squares). Cell extracts were subsequently assayed for CAT activity and data plotted as fold-activation relative to cells treated with vehicle (0.1% DMSO) alone. Data points represent the mean of assays performed in triplicate. Similar results were obtained in two independent experiments.

for nuclear receptors induce their interaction with proteins required for optimal transcriptional activation, the so-called coactivator proteins (Horwitz et al., 1996). These ligand-dependent interactions have been exploited as a biochemical assay for demonstrating direct interactions between ligands and their cognate receptors (Krey et al., 1997).

The steroid receptor coactivator protein-1 (SRC-1) has been shown to interact directly with both steroid

and nonsteroid nuclear hormone receptors in a ligand-dependent fashion (Onate et al., 1995; Horwitz et al., 1996; Kamei et al., 1996; Takeshita et al., 1996). The interaction of SRC-1 with nuclear receptors is dependent upon the amino acid motif LXXLL found in multiple copies in SRC-1 (Heery et al., 1997; Torchia et al., 1997). In an effort to determine whether the steroid activators of PXR serve as ligands for this orphan receptor, we tested whether a 14 kDa fragment of SRC-1 (SRC-1.14) containing three LXXLL motifs interacted with PXR in a steroid-dependent manner.

SRC-1.14 was expressed in vitro and labeled with [³⁵S]-methionine and [³⁵S]-cysteine, and the LBD of PXR.1 was expressed in *E. coli* as a fusion protein with glutathione-S-transferase (GST). Coprecipitation experiments were performed in the presence of the most potent PXR activators including 6,16 α -dimethyl pregnenolone, dexamethasone-t-butylacetate, and PCN. [³⁵S]-SRC-1.14 interacted only weakly with the GST-PXR.1LBD fusion protein in the absence of added compound (Figure 7A). The interaction of [³⁵S]-SRC-1.14 with GST-PXR.1LBD was significantly enhanced in the presence of either dexamethasone-t-butylacetate, 6,16 α -dimethyl pregnenolone, or PCN (Figure 7A). Additional experiments performed with 6,16 α -dimethyl pregnenolone and PCN revealed that these steroids promoted [³⁵S]-SRC-1.14/PXR.1LBD interactions in a dose-dependent manner (Figure 7B). Consistent with the results of the transfection studies, 6,16 α -dimethyl pregnenolone was more potent than PCN in promoting these interactions (Figure 7B). Estradiol did not stimulate interactions between PXR.1 and [³⁵S]-SRC-1.14, indicating that the enhancement was specific for compounds that activate PXR.1 in the transfection assay (Figure 7A). In control experiments, estradiol promoted the efficient interaction of [³⁵S]-SRC-1.14 with a GST-ER α LBD fusion protein (Figure 7A). However, GST-ER α LBD interactions with [³⁵S]-SRC-1.14 were not induced in the presence of PCN, dexamethasone-t-butylacetate, or 6,16 α -dimethyl pregnenolone. Taken together, these data provide strong evidence that PCN, dexamethasone-t-butylacetate, and 6,16 α -dimethyl pregnenolone serve as ligands for PXR.

Discussion

Identification of a Novel Signaling Pathway for Steroids

It was first observed over 50 years ago that repeated administration of high doses of certain steroids, including progestins and androgens, reduced their own and each other's toxic effects. These early observations, together with the later findings that resistance to numerous drugs is sex dependent, that castration of rodents leads to increased drug sensitivity, and that liver homogenates of intact male rats metabolize certain drugs more rapidly than homogenates prepared from castrated animals led to the concept of "catatoxic" steroids; that is, steroids that confer resistance to specific toxins by accelerating their metabolism (reviewed by Kourounakis et al., 1977). It was speculated that catatoxic agents might have utility in the treatment of patients suffering from either drug intoxication or from diseases caused by endogenous substances liable to metabolism (e.g., Cushing's syndrome) (Kourounakis et al., 1977).

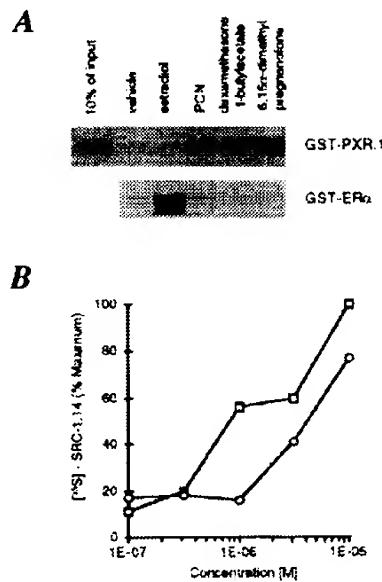


Figure 7. Steroids Induce PXR Interactions with a Fragment of the Coactivator Protein SRC-1.

(A) Coprecipitation experiments were performed with bacterially expressed GST-PXR.1LBD (upper panel) or GST-ER α LBD (lower panel) and [35 S]-SRC-1.14 synthesized in vitro. [35 S]-SRC-1.14 was mixed with either GST-PXR.1LBD or GST-ER α LBD in the presence of vehicle alone (1% DMSO) or 10 μ M of estradiol, PCN, dexamethasone, 1-butylacetate, or 6,16 α -dimethyl pregnenolone as indicated. [35 S]-SRC-1.14 complexed with either GST-PXR.1LBD or GST-ER α LBD was precipitated with glutathione-sepharose beads as described in Experimental Procedures. A lane representing 10% of the input [35 S]-SRC-1.14 in each reaction is shown on the left. Western blot analysis with an anti-GST antibody revealed that comparable amounts of GST-PXR.1LBD and GST-ER α LBD fusion proteins were used in the assays (data not shown).

(B) Dose-response analysis was performed with [35 S]-SRC-1.14 and GST-PXR.1LBD in the presence of the indicated concentrations of 6,16 α -dimethyl pregnenolone (squares) or PCN (circles). [35 S]-SRC-1.14 was quantitated via scanning densitometry and plotted as a percent of the [35 S]-SRC-1.14 precipitated in the presence of 10 μ M 6,16 α -dimethyl pregnenolone. Data shown represent the average of duplicate points, and similar results were obtained in two separate experiments.

A systematic analysis of steroids in the early 1970s identified the pregnenolone derivative PCN as the most potent cataotoxic compound among those tested (Selye, 1971). Insight into the mechanism underlying the cataotoxic effects of PCN was provided by the demonstration that this synthetic steroid induces the expression of CYP3A1 and CYP3A2, two closely related members of the P450 family of monooxygenases (Elshourbagy and Guzelian, 1980; Heuman et al., 1982; Hardwick et al., 1983; Schuetz and Guzelian, 1984; Schuetz et al., 1984; Gonzalez et al., 1985). The CYP3A hemoproteins have a remarkably broad substrate specificity, hydroxylating a variety of xenobiotics such as cyclosporin, warfarin, and erythromycin, as well as endogenous steroids including cortisol, progesterone, testosterone, and DHEA-sulfate (Nebert and Gonzalez, 1987; Juchau, 1990). Subsequent studies with the cloned CYP3A1 gene promoter identified a PCN response element that was highly conserved in the CYP3A2 gene promoter (Miyata et al., 1995;

Quattrochi et al., 1995). This response element was composed of two copies of the nuclear receptor half-site consensus sequence AGTTCA organized as a DR.

In addition to PCN, the expression of CYP3A1 was also shown to be induced by dexamethasone both in vivo and in cultured hepatocytes (Heuman et al., 1982; Schuetz and Guzelian, 1984; Schuetz et al., 1984). However, the concentrations of dexamethasone required to induce CYP3A gene expression were higher than those typically required to activate the classical GR signaling pathway (Schuetz and Guzelian, 1984; Schuetz et al., 1984). Promoter mapping studies showed that dexamethasone induced CYP3A1 gene expression through the same DR response element as PCN (Quattrochi et al., 1995; Huss et al., 1996). Thus, paradoxically, both high concentrations of dexamethasone, a glucocorticoid, and PCN, an antiglucocorticoid, induced the expression of the CYP3A1 gene through the same response element.

We now provide several lines of evidence indicating that the orphan nuclear receptor PXR is responsible for mediating the inductive effects of PCN and dexamethasone on CYP3A gene expression. First, both dexamethasone and PCN are efficacious activators of the PXR.1 isoform. Second, PXR binds efficiently as a heterodimer with RXR to the conserved DR-3 motifs identified in the CYP3A1 and CYP3A2 gene promoters as glucocorticoid and PCN response elements. Finally, we detected PXR expression in only a few tissues, including liver, intestine, and kidney. These are the primary tissues in which the CYP3A genes are expressed and induced in response to either dexamethasone or PCN treatment (Heuman et al., 1982; Debri et al., 1995). Our data thus support the existence of a novel signaling pathway for synthetic glucocorticoids and provide a mechanistic explanation for the long-standing paradox as to how both GR agonists and antagonists can exert similar effects on CYP3A gene expression. Moreover, the identification of PXR.1 as the PCN receptor provides the tool necessary for the rapid identification of novel pharmacological agents with more potent cataotoxic activities.

In addition to inducing CYP3A gene expression, PCN is also known to have marked effects on hepatic cholesterol homeostasis in rodents. These effects include significant decreases in the levels of HMG-CoA reductase and cholesterol 7 α -hydroxylase gene expression with concomitant reductions in sterol biosynthesis and bile acid secretion (von Bergmann et al., 1975; Mason and Boyd, 1978; Turley and Dietschy, 1984; Stahlberg, 1995). PCN has also been reported to enhance the formation of cholesterol-esters and the hypersecretion of cholesterol into the bile (von Bergmann et al., 1975; Turley and Dietschy, 1984). Thus, PCN affects key aspects of cholesterol metabolism including its biosynthesis, storage, and secretion. Although we cannot exclude the possibility that some of its biological effects might be mediated through the GR or other steroid receptors, it is tempting to speculate that PCN is mimicking the actions of an endogenous hormone that serves to regulate coordinately steroid and sterol metabolism through the activation of PXR in tissues such as liver and intestine. Our data raise the possibility of the existence of regulatory loops through which endogenous PXR hormones feed back to regulate cholesterol homeostasis and feed forward to regulate steroid homeostasis.

What is the natural ligand for PXR? Pregnanolone and progesterone are among the most potent naturally occurring PXR activators that we have identified, activating PXR.1 at low micromolar concentrations. Pregnanolone is one of the most abundant steroids in mammals, circulating in humans and rodents at concentrations that range from roughly 1 to 50 nM (Punjabi et al., 1983; Wichmann et al., 1984; Tietz, 1995). While progesterone levels in human serum are generally in the 1–10 nM range, concentrations can exceed 700 nM during the third trimester of pregnancy (Tietz, 1995). These levels approach those required to activate PXR.1 *in vitro*. Nevertheless, it remains unclear whether concentrations of progesterone or pregnanolone sufficient to activate PXR.1 are achieved either in serum or in tissues under normal physiological conditions. Our results with PXR may thus be analogous to those obtained with RXR, which was first shown to be activated by micromolar concentrations of all-trans retinoic acid (t-RA) prior to the identification of 9-cis RA as a high-affinity ligand (Mangelsdorf et al., 1990; Heyman et al., 1992; Levin et al., 1992). Based upon our findings that PXR is activated by pregnanolone and its metabolites and that the synthetic steroid 6,16 α -dimethyl pregnenolone activates PXR with an EC₅₀ value of 300 nM, we suggest that the natural PXR ligand is likely to be a pregnane.

Perspectives

With the isolation of the androgen receptor in 1988 (Chang et al., 1988; Lubahn et al., 1988), receptors for all of the known nuclear-acting steroid hormones had been cloned. However, studies performed during the past two years with orphan members of the nuclear receptor family have suggested that additional sterols are likely to serve as mammalian hormones. For example, the orphan receptors LXR and SF-1 were recently shown to be activated by physiological concentrations of several oxysterol metabolites of cholesterol (Janowski et al., 1996; Lala et al., 1997; Lehmann et al., 1997). While the biological role of LXR remains less clear, SF-1 is essential for adrenal and gonadal development and regulates the expression of genes required for steroidogenesis (Luo et al., 1994). These data suggested an unexpected hormonal function for oxysterols in the regulation of steroidogenesis.

We have now identified PXR as a novel member of the nuclear receptor family that is efficaciously activated by both natural and synthetic steroids. The activation profile of PXR is distinct from any of the other steroid hormone receptors identified to date, suggesting that this orphan receptor defines a novel endocrine signaling pathway. We conclude that the identification of PXR provides additional evidence for an expanded role for steroid hormones in mammalian physiology and that the elucidation of the biological role of PXR is likely to lead to a better understanding of how steroids elicit their myriad effects.

Experimental Procedures

Chemicals

Dexamethasone-t-butylacetate and RU486 were purchased from Research Plus, Inc. (Bayonne, NJ) and Biomol (Plymouth Meeting,

PA), respectively. All other steroids were purchased from either Sigma Chemical Co. (St. Louis, MO) or Steraloids, Inc. (Wilton, NH).

Molecular Cloning of PXR cDNAs

Partial mouse sequence for PXR was obtained from an EST deposited in the Washington University/HHMI EST database (accession number AA277370). A 19 bp oligonucleotide derived from this EST sequence (5' TCTCCCCAGATCGTCCTGG 3') was used to screen a pCMV-SPORT mouse liver cDNA library (GIBCO-BRL) using Gene Trapper solution hybridization cloning technology (GIBCO-BRL). Five clones were obtained that ranged in size from 1.0 kb to 2.7 kb. Four of these clones encoded PXR.1 and one encoded the DBD and LBD of PXR.2. A clone encoding the full-length PXR.2 isoform-coding region (nucleotides 151 to 1443) was subsequently isolated from mouse liver cDNA through PCR. Sequences were aligned and analyzed by the University of Wisconsin Genetics Computer Group programs.

Plasmids

The expression plasmids pSG5-GAL4-PXR.1LBD and pSG5-GAL4-PXR.2LBD were generated by amplification of cDNA encoding amino acids 105–431 of PXR.1 or 105–390 of PXR.2 by PCR and insertion into a modified pSG5 expression vector (Stratagene) containing DNA encoding the DBD of GAL4 (amino acids 1–147) and the SV40 Tag nuclear localization signal (APKKRKVGV) inserted upstream of a multiple cloning site. The (*UAS*)-*tk*-CAT reporter plasmid has been previously described (Lehmann et al., 1995). The expression vectors pSG5-PXR.1 and pSG5-PXR.2 were generated by amplification of cDNA encoding amino acids 1–431 of PXR.1 or 1–390 of PXR.2 and insertion into pSG5. The reporter plasmid (*CYP3A1*)-*tk*-CAT was generated by insertion of two copies of a double-stranded oligonucleotide containing the *CYP3A1* DR-3 RE (5' GATCAGACAGTTCATGAAGTTCATCTAGATC 3') (Quattrochi et al., 1995; Huss et al., 1996) into the BamHI site of pBLCAT2 (Luckow and Schötz, 1987). The bacterial expression vector pGEX-PXR.1LBD was generated by PCR amplification of cDNA encoding amino acids 105–431 of PXR.1 and insertion into a pGEX-2T vector (Pharmacia) that had been modified to contain KpnI and NotI restriction sites. The bacterial expression vector pGEX-ER α LBD was generated by PCR amplification of cDNA encoding amino acids 251–595 of human ER α (Green et al., 1986) and insertion into pGEX-2T. The expression plasmid for the SRC-1.14 fragment was generated by PCR amplification of DNA encoding amino acids 632–754 of human SRC-1 (Takeshita et al., 1996) and insertion into the expression vector pRSETC (Invitrogen). All constructs were confirmed by sequence analysis.

Cotransfection Assays

CV-1 cells were plated in 24-well plates in DME medium supplemented with 10% charcoal-stripped fetal calf serum at a density of 1.2 \times 10⁴ cells per well. In general, transfection mixes contained 33 ng of receptor expression vector, 100 ng of reporter plasmid, 200 ng of β -galactosidase expression vector (pCH110, Pharmacia), and 166 ng of carrier plasmid. Cells were transfected overnight by lipofection using Lipofectamine (Life Technologies, Inc.) according to the manufacturer's instructions. The medium was changed to DME medium supplemented with 10% defatted calf serum (Sigma) and cells were incubated for an additional 24 hr. Cell extracts were prepared and assayed for CAT and β -galactosidase activities as described previously (Lehmann et al., 1995).

Northern Analysis

An approximately 1.0 kb fragment encoding the LBD of PXR.1 (nucleotides 463–1446) was ³²P-labeled by random priming using the T7 Quick-Prime kit (Pharmacia). This radiolabeled fragment was used to probe mouse multiple-tissue Northern blots (OriGene, Rockville, MD). Blots were hybridized in ExpressHyb solution (Clontech, Palo Alto, CA) at 65°C for 18 hr. Final washes were performed with 0.1 \times SSC, 0.1% SDS at 65°C.

In Situ Hybridization Analysis

Embryonic day 18 (E18) mice (C57Bl/CBA and NMRI, Bornholts Breeding and Research Center, Copenhagen, Denmark) were used in the studies. Tissues were sectioned at 14 μ m and thaw-mounted

onto slides (ProbeOn, Fischer). Three different oligonucleotide probes (Pharmacia Biotech, Solna, Sweden) designed to hybridize to *PXR* mRNA were used. Oligonucleotides were radiolabeled using [³⁵S]-deoxyadenosine 5'- α -thio-triphosphate (NEN) at the 3' end using terminal deoxynucleotidyl transferase (Amersham). Labeled oligonucleotides were hybridized to tissue sections and mRNA expression was first detected by phosphoimaging (BASF 3000 Phosphoimager, Fuji) followed by film emulsion autoradiography (Dagerlind et al., 1992). Sections were examined using light- and dark-field microscopy (Axiphot, Zeiss) and photographed (Ektachrome 64T, Kodak). Positives were scanned (SprintScan 35, Polaroid) and processed using Photoshop and PageMaker, Adobe. The following oligonucleotide sequences were used as probes: probe 1 (*PXR*.1 and *PXR*.2 specific), 5' GGAGCCTCAATCTTTCC TCTCTTCCTTGATCAAGGCCCG 3'; probe 2 (*PXR*.1 specific), 5' CTTCCAACAGTGAGGCTGCAGAAACTGGAAAGCTCACAGC CAC 3'; probe 3 (*PXR*.1 and *PXR*.2 specific), 5' TGGGCTCTTCAAAG GCAGAGTCTGCTTCT TCACACTGTACAAGGCC 3'. A 50 bp random oligonucleotide was used as a negative control.

Gel Mobility Shift Assays

PXR.1, *PXR*.2, and *RXR* α were synthesized in vitro using the TNT rabbit reticulocyte lysate coupled in vitro transcription/translation system (Promega) according to the manufacturer's instructions. Gel mobility shift assays (20 μ l) contained 10 mM Tris (pH 8.0), 40 mM KCl, 0.05% NP-40, 6% glycerol, 1 mM DTT, 0.2 μ g of poly(dI-dC), and 2.5 μ l each of in vitro-synthesized *PXR* and *RXR* proteins. The total amount of reticulocyte lysate was maintained constant in each reaction (5 μ l) through the addition of unprogrammed lysate. Competitor oligonucleotides were included at a 5-fold or 25-fold excess as indicated in the figure legends. After a 10 min incubation on ice, 10 ng of ³²P-labeled oligonucleotide was added and the incubation continued for an additional 10 min. DNA-protein complexes were resolved on a 4% polyacrylamide gel in 0.5 \times TBE (1 \times TBE = 90 mM Tris, 90 mM boric acid, 2 mM EDTA). Gels were dried and subjected to autoradiography at -70°C. The following double-stranded oligonucleotides were used as either radiolabeled probes or competitors: *CYP3A1*, 5' GATCAGACAGTTCATGAAGTCATCT AGATC 3' (Quattrochi et al., 1995); Δ *CYP3A1*, 5' GATCAGACAGAAC ATGAGAACATCTAGATC 3'; *CYP3A2*, 5' GATCAAACAGTTCATAA AGTTCATCTAGATC 3' (Miyata et al., 1995).

SRC-1.14 Coprecipitation Assay

GST-*PXR*.1LBD and GST-*ER* α LBD fusion proteins were expressed in BL21(DE3)plysS cells and bacterial extracts prepared by one cycle of freeze-thaw of the cells in protein lysis buffer containing 10 mM Tris (pH 8.0), 50 mM KCl, 10 mM DTT, and 1% NP-40 (followed by centrifugation at 40,000 \times g for 30 min). Glycerol was added to the resulting supernatant to a final concentration of 10%. Lysates were stored at -80°C. [³⁵S]-SRC-1.14 was generated using the TNT rabbit reticulocyte system (Promega) in the presence of Pro-Mix (Amersham). Coprecipitation reactions included 25 μ l of lysate containing GST-*PXR*.1LBD or GST-*ER* α LBD fusion proteins or control GST; 25 μ l incubation buffer (50 mM KCl, 40 mM HEPES [pH 7.5]; 5 mM β -mercaptoethanol; 1% Tween-20, 1% non-fat dry milk); 5 μ l [³⁵S]-SRC-1.14; and either PCN; dexamethasone-1-butylacetate; 6,16-dimethyl pregnenolone; estradiol; or control DMSO. The mixtures were incubated for 25 min at 4°C with gentle mixing prior to the addition of 15 μ l of glutathione-sepharose 4B beads (Pharmacia) that had been extensively washed with protein lysis buffer. Reactions were incubated with gentle mixing at 4°C for an additional 20 min. The beads were pelleted at 2000 rpm in a microfuge and washed 3 times with protein lysis buffer containing either PCN; dexamethasone-1-butylacetate; 6,16-dimethyl pregnenolone; estradiol; or control DMSO. After the last wash, the beads were resuspended in 25 μ l of 2 \times SDS-PAGE sample buffer containing 1 mM DTT. Samples were heated at 100°C for 5 min and loaded onto a 10% Bis-Tris PAGE gel. Autoradiography was performed for 2-4 days. Assays were quantitated using a Molecular Dynamics Computing Densitometer and Image Quant software.

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GenBank Accession Number

The PXR sequence has been deposited in GenBank (accession number AF031814).